Chapter **V**

The Absence of Early Calcium Response to Heavy Ion Irradiation in Mammalian Cells

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Running Title: Absence of Calcium Response to Ion Irradiation

Du, G., Fischer, B.E., Voss, K.O., Becker, G., Taucher-Scholz, G., Kraft, G. and Thiel, G. The Absence of Early Calcium Response to Heavy Ion Irradiation in Mammalian Cells. *Radiat. Res.*

Abstract

Intracellular Calcium is an important second messenger regulating many cell functions. Recent publications have shown that Calcium ions can also regulate the cellular responses to ionizing radiation. However, previous data are restricted to cells treated with low LET radiations (X-rays, γ-rays and β-rays). In this work, we investigated the Calcium levels in cells exposed to heavy ion radiation of high LET. The experiments were performed at the single ion hit facility of the GSI heavy ion microprobe. Using an inbuilt online Calcium imaging system, the intracellular Calcium concentration was examined in HeLa cells and human foreskin fibroblast AG1522-D cells before and after irradiation with 4.8 MeV/nucleon Carbon or Argon ions. Although the experiment was sensitive enough to detect the Calcium response to other known stimuli, no response to ion irradiation was found in these two cell types. Meanwhile, we also demonstrated that ion irradiation has no impact on Calcium oscillation induced by hypoxia stress in fibroblast cells.

Introduction

Cytoplasmic Calcium is one of the most important second messengers in eukaryotic cells. The concentration of the free Ca^{2+} in the cytoplasm ($[Ca^{2+}]_{cyt}$) is only in the order of some 100 nM but it reaches millimolar concentrations at the external side of the cells and in many organelles such as in the endoplasmic reticulum (ER) and the mitochondria. In the case of a relevant stimulus $[Ca^{2+}]_{cyt}$ increases either by Calcium mobilization from the intracellular Calcium store or by Calcium influx via Ca^{2+} channels in the plasma membrane. Also a combination of both mechanisms is relevant (*1*). Recent studies have reported that $[Ca^{2+}]_{cyt}$ may play important roles in regulating several cellular responses to ionizing radiation. These include cell cycle arrest (*2*), apoptotic DNA fragmentation (*3-4*), γ-GCS mRNA expression (*5*) and DNA repair (*6*). It was also reported that $[Ca^{2+}]_{cyt}$ can be affected in several different cell lines exposed to X-, γ- and β-radiation. These radiation-induced changes of $[Ca^{2+}]_{cyt}$ occurred in the form of elevations, oscillations or single transient changes within minutes to days after irradiation (*3-4, 7-9*).

Protons and Carbon ions, which have been successfully used to treat tumor/cancer patients in several countries (*10*), have not yet been investigated in the context of their influence on Ca^{2+} signaling. These ions have a linear energy transfer (LET) of ~100 keV/μm, which is 100 times higher than the LET of X-, γ- and β-radiation. Therefore, it is worthwhile to investigate the role of Calcium signaling in the early cellular response to ion irradiation.

A new experimental approach for addressing radiation induced $[Ca^{2+}]_{cyt}$ changes at the cellular level is provided by a microbeam facility at GSI (*11*). It allows delivery of ions spanning from Helium to Uranium in sub-cellular regions of individual cells with defined doses (ion hits) (*12*). In the present study, we combined this microbeam facility with a newly developed Calcium imaging setup. The combination of the two devices allowed monitoring of intracellular Calcium in living cells at high spatial and temporal resolution before and immediately after heavy ion irradiation. Using this system, we examined $[Ca^{2+}]_{cyt}$ in HeLa cells and human fibroblast cells in response to Carbon and Argon ions. We report here that $[Ca^{2+}]_{cyt}$ in both cell types was not affected within a time window of 30 minutes after irradiation.

Materials and Methods

Chemicals and solutions

Culture medium MEM-EARLE (containing 1% L-Glutamine), culture medium RPMI 1640 (without phenol red), fetal calf serum (FCS), L-Glutamine, PBS and 1M HEPES solution were purchased from Biochrom AG (Germany). Culture medium EMEM with EBSS was purchased from BioWhittacker (Belgium). Fura-2/acetoxymethyl ester (Fura-2/AM), Mouse monoclonal IgG anti phosphohistone H2AX (ser-139) and Anti-mouse IgG-AF488 were purchased from Invitrogen/Molecular Probes (Germany). Propinium Iodide (PI) and RNase were purchased from Applichem (Germany). Bovine serum albumin (BSA) was purchased from Carl Roth GmbH (Germany). The Trypsin used for cell passage was purchased from PAN Biotech GmbH (Germany).

Fura-2/AM was solved in pure DMSO (Sigma-Aldrich, Germany) to prepare a 0.5 mM Fura-2/AM stock solution. Twenty mM HEPES medium buffer was prepared by dilution of 1M HEPES in corresponding culture medium with a final pH of 7.5. Two mM Hydrogen peroxide solution was prepared by dilution of 10 M H_2O_2 (Sigma-Aldrich, Germany) in 20 mM HEPES buffer; this was used to treat the cells to test the performance of the Calcium imaging system.

Cell culture

Human cervix epitheloid carcinoma HeLa cells (a gift from Dr. Nuri Gueven, The Queensland Institute of Medical Research, Australia) were cultured in a culture flask as a monolayer in MEM-EARLE medium plus 10% FCS or in RPMI 1640 medium plus 10% FCS and 1% L-Glutamine at 37 °C, (100% humidity, 95% air/5% CO_2). Human foreskin fibroblast AG1522-D cells (Coriell Cell Repository, Camden, NJ, USA) were cultured confluent in EMEM with EBSS plus 15% FCS, 1% stable glutamine and 1% Penicillin/Streptomycin at 37 °C (100% humidity, 95% air/5% CO_2). AG1522-D cells were transferred into a Glutamine-free medium two weeks prior to the experiment.

A home-made cell dish is used for cell irradiation at the single ion hit facility. The cell dish is made of stainless-steel with a culture bottom of 4 µm thick Polypropylene foil

(not coated with Cell-Tak, for further details about the cell dish see ref. *12*). Two or three days before the experiment, HeLa cells were removed from culture flask by trypsinization and seeded with a density of 500 cells per cm^2 in the cell dishes. One day or two days before the experiment, AG1522 cells were trypsinized and seeded with a seeding density of 2000 cells per cm^2 in the cell dishes. Both AG1522-D (CPD 21-25) cells and HeLa cells attached and grew well in the cell dishes with the seeding-densities used.

Fura-2/AM loading

HeLa cells and AG1522-D cells were loaded in 2.5 μM Fura-2/AM solution (0.5mM Fura-2/AM diluted in 20mM HEPES buffer) at 37°C for 45 min; then rinsed three times with 20mM HEPES buffer and kept in this buffer for de-esterification at room temperature for 30 minutes.

Experimental system

Cell irradiations have been performed at the single ion hit facility at GSI since 2003. Briefly, the facility uses a magnetic quadrupole triplet lens to focus the ion beam into a spot of less than 1 μm diameter, and it can deliver a counted number of ions into sub-cellular regions of individual cells with a positioning accuracy of 1.7 μm. As shown in Fig. 1, the Calcium imaging system is added behind the cell stage. It is composed of a filter wheel with two excitation filters (a 380 nm filter, cat. 380FS10-12.5 from LOT-Oriel, EU; and a 340 nm filter, cat. FF01-340/26 from Semrock, USA), an emission filter (525 nm filter, cat. HQ525/50 from Chroma Technology Corp., USA), a CCD camera (Pixelfly, PCO AG, Germany). As the 340 nm filter and the emission filter can also transmit infra-red light, an infra-red blocker is also included in the light path in order to block the infra-red light from the UV lamp. Otherwise, the infra-red light reflected by the vacuum window would have corrupted the fluorescence imaging. To minimize the UV exposure and avoid photo bleaching, we attenuated the excitation light to the lowest intensity required using a neutral density filter. Also, a light shutter in the UV light path was only opened during fluorescence imaging. The system acquired the fluorescence images excitated at 340 nm and 380 nm with an exposure time of 200 or 400 ms per image and a frequency of 2-10 pairs of image per minute.

Irradiation and Calcium measurement

After the Fura-2 loading, the cell dishes were closed with two types of covers before the irradiation, namely, some dishes were closed with cover glass as in former experiments (labeled as standard cell dishes) (*12*), others were closed with gas permeable foil (bioFOILIE25, In Vitro Systems & Services, Germany) (labeled as cell dishes with increased gas exchange). Then the dishes were inserted vertically into the cell stage of the single ion hit facility. Once the target cells were selected manually, the measurement started with a three-minute basal ratio-imaging, then the cells were irradiated with defined numbers of 4.8 MeV/nucleon Carbon or Argon ions by hitting the nucleus or the cytoplasm. Depending on the number of ion hits delivered per field of view, the irradiation took several seconds to tens of seconds. The measurement

continued for another 30 minutes immediately after the irradiation (the total experiment takes 34 minutes). Due to the heating from the magnetic lens, the temperature of the cell dish was 29°C during the experiment. The fluorescence images were subsequently analyzed cell by cell offline. The fluctuation of the UV lamp was compensated and the background of the fluorescence images was subtracted by using an area without cell in the fluorescence images. As shown in Fig.3a, a specific part of the cell can be chosen as an area of interest (AOI). The mean fluorescence intensities of the AOI excited by 380 nm and 340 nm (F380 and F340) were extracted and the ratio R=F340/F380 was used as the indicator of Calcium concentration (*13*).

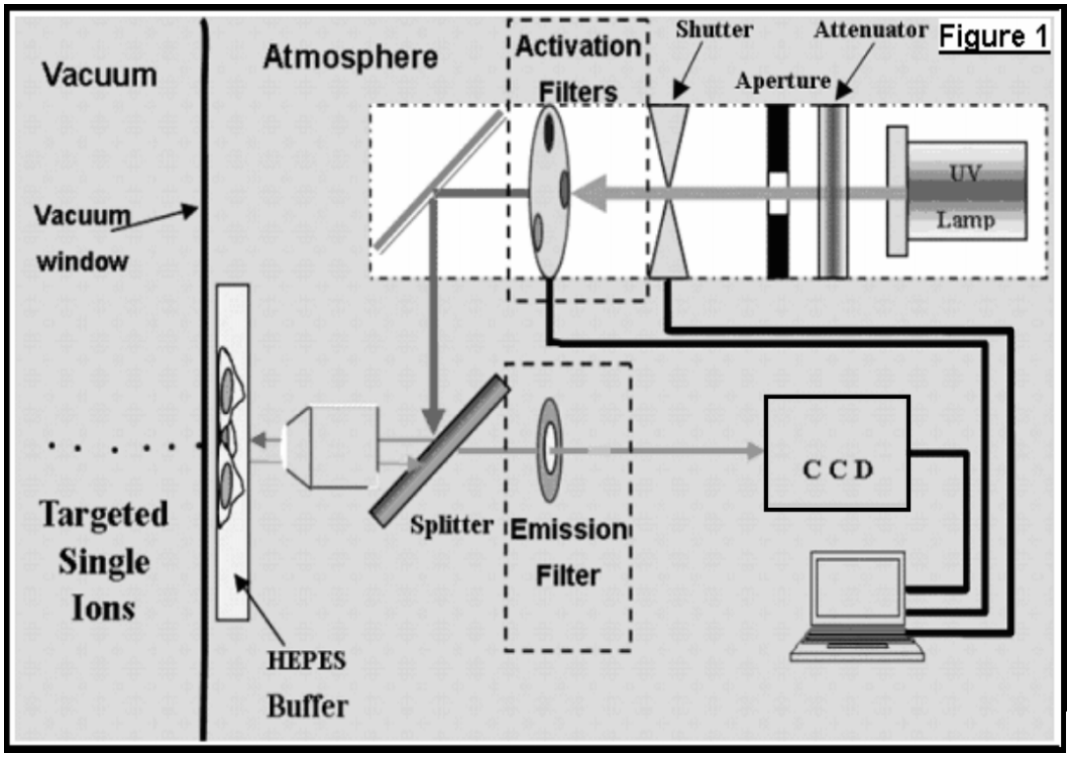


Fig.1 Schematic of the ratio imaging setup added to the single ion hit facility at GSI.

Verification of ion hits by immuno-fluorescence microscopy

To test whether the cells were indeed hit by ions as expected, we examined the γ-H2AX immuno-fluorescence which can be used to visualize ion hits in the cell nucleus (*14*). As this method also reveals foci in part of the cells without irradiation, we usually irradiated the cells with a cross pattern of hits (3 or 5 points per axis, 2 or 3 μm between two points). These cross patterns can be clearly identified as real hits because of their artificial shape (*12*). Immediately after the Calcium measurement HeLa and AG1522-D cells were fixed in 2% Paraformaldehyde for 20 minutes at room temperature, permeabilized by with Triton X-100 (0.5% v/v in PBS) for 7 minutes, rinsed 3 times using PBS and blocked with BSA (0.5% w/v in PBS). Then the fixed cells were incubated with γ-H2AX antibody (Mouse monoclonal IgG anti

phosphohistone H2AX (ser-139)) and secondary antibody (Anti-mouse IgG-AF488). After the immunostaining the cells were treated with RNase and the cell nuclei were counter-stained with PI. The prepared cells were observed using a Leica TCS confocal system equipped with a DM IRBE inverted microscope (20x and 63x objectives).

Results

Test of the Calcium ratio imaging setup with the known stimulus H_2O_2

To assess the performance of the ratio imaging setup integrated into the single ion hit facility, AG1522-D cells were treated with 2 mM H_2O_2 medium. In a time course measurement, the ratio F340/F380 increased immediately upon H_2O_2 treatment and remained at an elevated level as shown in Fig.2. Similarly, such a H_2O_2 treatment also induced detectable but relatively smaller changes of the fluorescence ratio in HeLa cells (data not shown).

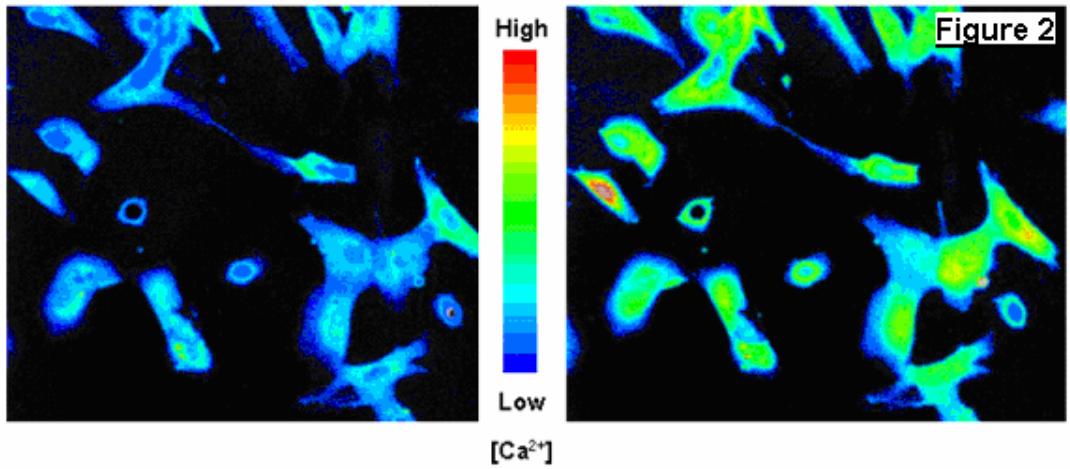


Fig. 2 Ratio images showing the elevation of $[Ca^{2+}]_{cyt}$ in AG1522-D cells induced by 2 mM hydrogen peroxide(20x, F340/F380, pseudo color). Left: before the treatment; right: immediately after the treatment.

Hit verification

Fig. 3 shows that the single ion hit facility allows an accurate positioning of the ion hits. Only the selected cells in Fig. 3a (marked with red crosses) will be hit by ions at the desired positions, i.e. this allows targeting the cytoplasm and/or the nucleus within a single cell. Concurrently, other cells in the same culture dish can be spared from ion irradiation and used as non-irradiated bystander cells. Some of these cells are in direct contact to the irradiated cells; these cells can be monitored in order to investigate a cell-to-cell signaling via gap junctions. With these features the setup provides two experimental advantages over previous studies: First, it is a means of monitoring the same cell before and immediately after radiation at a defined area of the cell. Secondly the ability to monitor the response of both the cells exposed to ion

irradiation and other cells in the same field of view eliminates differences in sample operation and experimental environment between irradiated cells and non-irradiated cells; such a cell to cell variability is generally encountered when comparing populations of cells.

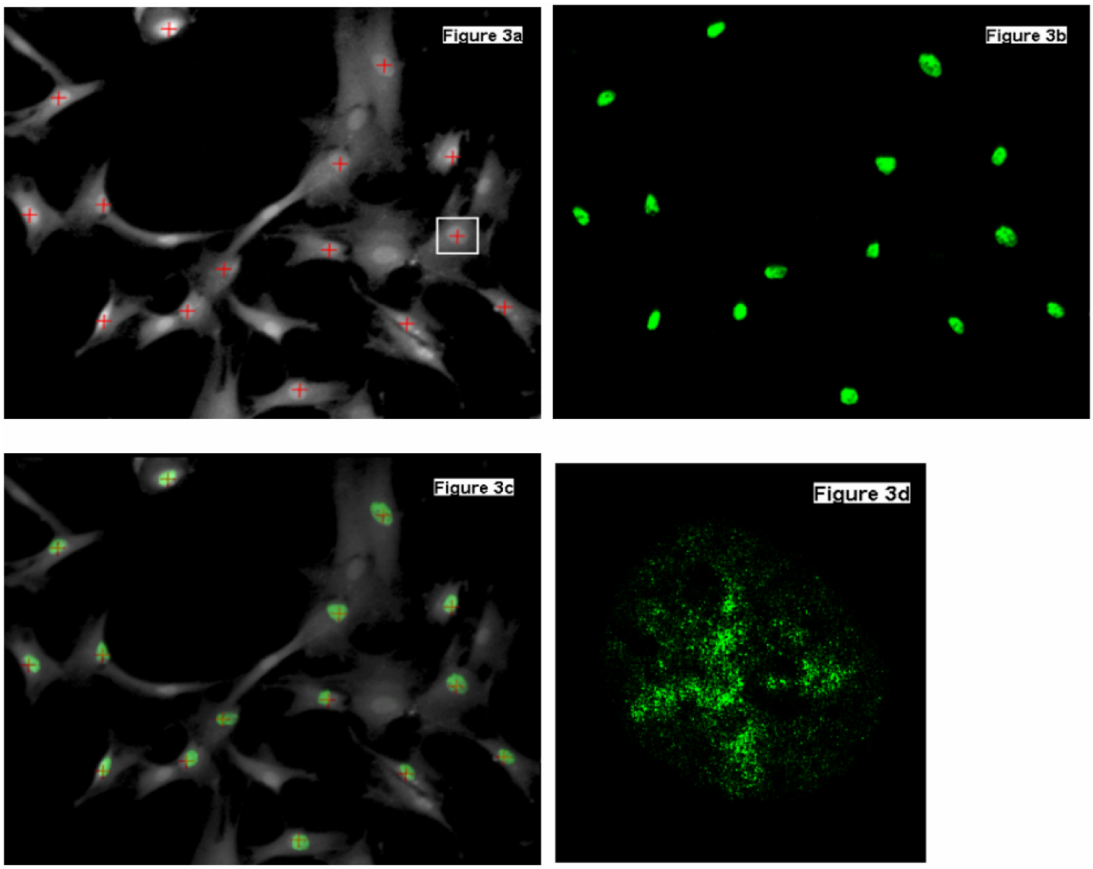


Fig. 3 Targeted ion irradiation and hit verification in AG1522-D cells. (a) Typical fluorescence image (20x) of AG1522-D cells loaded with Fura-2/AM. The red crosses mark the cells to be irradiated. Normally only the cell nucleus, in some samples only the cell plasma, is chosen as a target for ion hits. The non-marked cells are used as non-irradiated bystander controls. (b) Confocal microscopic image (20x) of the same field of view as in (a). The sample was fixed immediately after the Calcium recording (ca. 35 minutes pos-irradiation) and then immuno-stained by Gamma-H2AX antibodies. (c) Merge of image (a) and (b), the Gamma-H2AX foci coincide with the cell nuclei marked with a cross in (a). (d) Confocal microscopic image (63x) of the cell nucleus marked with a white rectangle in (a) reveals the cross pattern of ion hits and verifies the accurate targeting of the single ion hit facility.

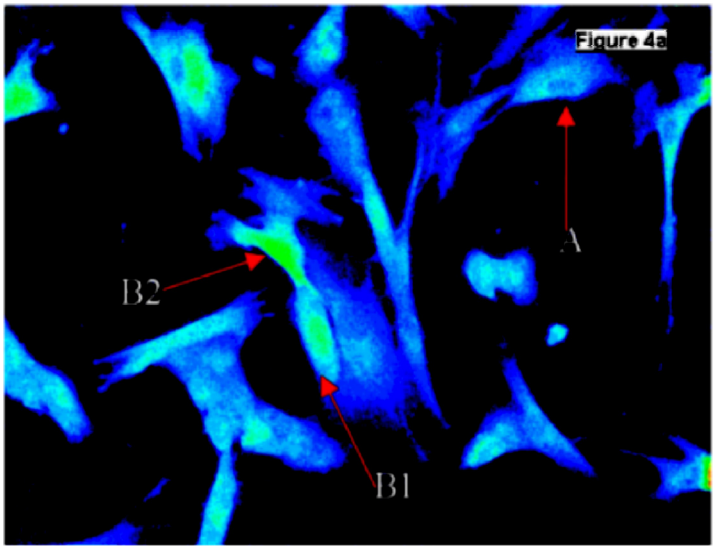
To test whether or not cells were indeed hit by ions we removed the same culture dish, which was used for $[Ca^{2+}]_{cyt}$ recordings (see result below) and fixed the cells for immuno-staining. Fig.3b shows a confocal microscopic image of the same cells as in 3a, which are now immuno-stained with γ-H2AX antibody. This antibody labels γ-

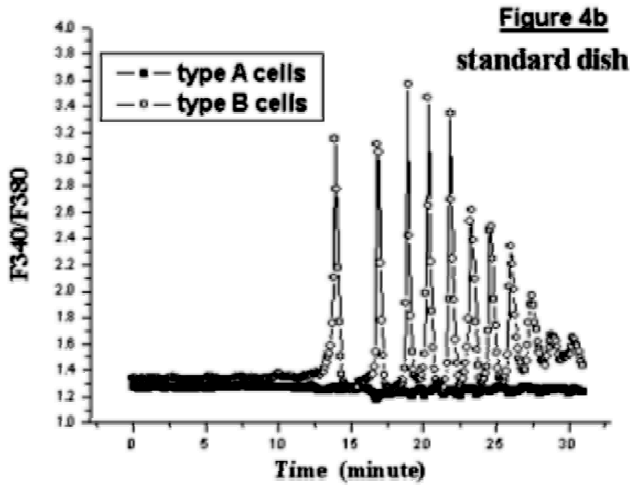
H2AX foci in the cell nucleus forming in response to DNA double-strand break (DSB) (*15*). As shown in Fig. 3c, the immuno-stained cells coincide well with the targeted cells. Even the cross shape of the ion target can be recognized at higher magnification in Fig. 3d. This means that the ions precisely hit the nucleus in the well defined positions; because of the accuracy of the targeting we can be assured that the cytoplasm was also successfully hit by ions in experiments when the cytoplasm was targeted.

Calcium effect is sensitive to environmental stress

In control experiments, $[Ca^{2+}]_{cyt}$ was monitored in fibroblast cells kept in standard cell dishes. As shown in Fig. 4, in these control experiments already unirradiated cells revealed a complex behavior: around 26% of the cells (27 cells out of 105 cells, 4 experiments) kept a stable resting $[Ca^{2+}]_{cyt}$ level during the entire (30 min) period of the experiment (type A cells in Fig. 4a and 4b, see the movie in supplemental materials); 67% percent of the population (71 cells out of 105 cells) showed Calcium oscillations (type B cells in Fig. 4a and 4b). These oscillations started in all cases ca. 15 minutes after the start of the experiment. Only in a minority (ca. 7 %) of cells Ca^{2+} was oscillating spontaneously during the entire period of recording (not shown). In some cases it was found that neighboring cells oscillated in a highly concerted fashion. An example is shown in Fig. 4c where the Ca^{2+} oscillations of the cells B1 and B2 from Fig. 4a are superimposed. Such a synchronization, which is only seen in neighboring cells, implies that some cell pairs are indeed coupling via gap junctions (see movie in supplemental materials).

In further experiments, we examined the reason for these spontaneous oscillations. We ruled out that the oscillations were due to the long-time UV exposure or the heating from the magnetic lens, because spontaneous oscillations were also observed in areas not exposed to UV light and after turning off the lens. The fact that the oscillation started after some time led us to suspect that the cover glass, which was used to close the standard cell dish, limited the supply of oxygen in the cell dish, and the oscillations could be caused by hypoxia stress (*16*). To test this hypothesis we repeated the control experiments using the cell dishes with increased gas exchange. Under these conditions nearly all the fibroblast cells (70 out of 72 cells from 3 experiments) did not show any more spontaneous Calcium oscillation (data not shown). Hence the spontaneous oscillations can be interpreted as a response to hypoxia-stress.





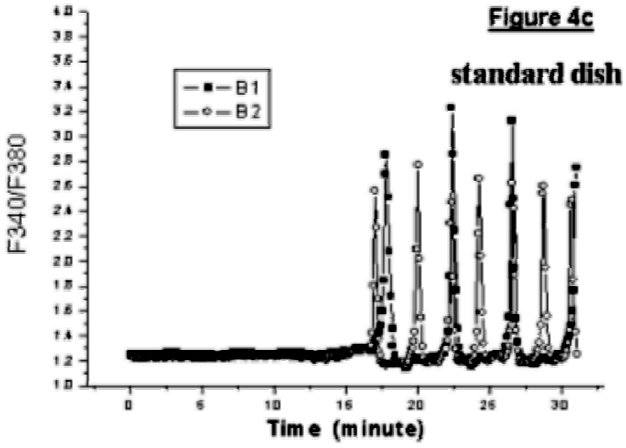


Fig. 4 Stress-induced Calcium signaling in control AG1522-D cells. (a) Ratio image (20x) of F340/F380 taken during the measurement. (b) Typical measurement of ratio (F340/F380) as a function of time in the standard cell dish; in 4 independent experiments around 26% (27 out of 106) of the population kept a stable resting $[Ca^{2+}]_{cyt}$ level during the entire (30 min) period of the experiment (type A cells). 67% (71 out of 106) of the population showed Calcium oscillations (Type B cells), which started in all cases ca. 15 minutes after the start of the experiment. A minority (ca. 7 %) of the cells $[Ca^{2+}]_{cyt}$ was oscillating spontaneously during the entire period of recording; such cells were excluded from analysis in further experiments because they showed oscillation before the ion irradiation. (c)

No immediate Calcium response to heavy ion irradiation in AG1522 and HeLa cells

To exclude the stress of hypoxia on the cellular response we first examined the Ca^{2+} response in AG1522-D cells in cell dishes with increased gas exchange. Cells kept under these conditions were hit by 144 Argons ions at the region of the nucleus. As shown in the exemplary cells in Fig. 5a, all cells which were "shot" and definitely hit with Argon ions (see Fig. 3) remained at a stable Calcium level during the entire 30 minutes and even an exposure to a dose of about 130 Gy of 4.8 MeV/nucleon Argon induced no perceivable effect on $[Ca^{2+}]_{cyt}$ after the irradiation. The same results were obtained in 3 independent experiments with 76 irradiated cells analyzed. Also in the non-irradiated bystander AG1522-D cells no perceivable change of $[Ca^{2+}]_{cyt}$ was found in all the experiments.

Furthermore, we also investigated the influence of ion irradiation on cells under hypoxia stress. Because Ca^{2+} oscillation is initiated by a complex signaling cascade involving calcium induced calcium release (*17*), it is possible to examine the effect of radiation on this signaling cascade. Fig. 5b shows a plot of two typical AG1522-D cells before and after ion-radiation in the standard cell dishes. The data show that irradiation had no perceivable effect on the resting $[Ca^{2+}]_{cyt}$ concentration in the time window when cells were generally not oscillating. The same result was obtained when analyzing 153 other cells in a total of 8 independent experiments in which cells were irradiated either in the nucleus and/or the cytoplasm and in which the dose varied from 2.5 to 50 Gy.

The data in Fig. 5c also show that the irradiated AG1522-D cells exhibit a similar behavior to those in the control experiment without any irradiation in that the cells started to oscillate spontaneously after some time (Fig. 4). A detailed statistical comparison of irradiated and control cells (in Fig. 6) shows that irradiation did neither increase nor decrease the percentage of the oscillating cells. Furthermore an analysis of the $[Ca^{2+}]_{cyt}$ oscillations reveals no significant difference between control cells, non-irradiated bystander cells and irradiated cells with respect to onset, amplitude and frequency of the oscillations. This means that ion irradiation has neither positive nor negative influence on any of the steps contributing to the complex mechanism of $[Ca^{2+}]_{cyt}$ oscillations.

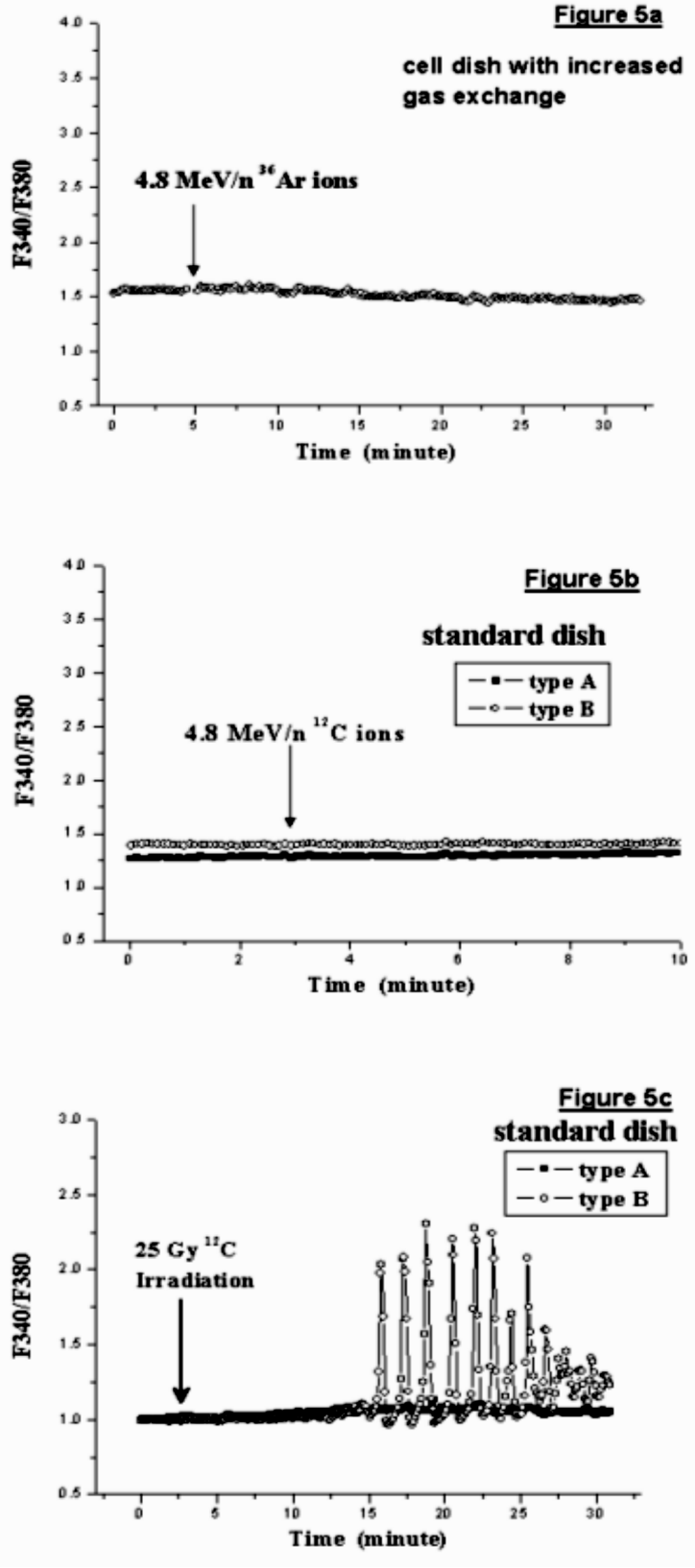


Fig. 5 Heavy ion irradiation did not trigger and also not inhibit calcium signaling in AG1522-D cells. (a) The cells in the standard cell dishes showed no change of $[Ca^{2+}]_{cyt}$ level in the defined time window after Carbon ion irradiation (180 ion hits targeted at nucleus ~ 47Gy). (b) The cells in the gas permeable cell dish kept a stable $[Ca^{2+}]_{cyt}$ level before and after Argon ion irradiation (144 ion hits targeted at nucleus ~130 Gy). (c) Ion irradiation did not inhibit the stress-induced Calcium oscillation. The arrow indicates the time point at which the irradiated cells were hit by heavy ions.

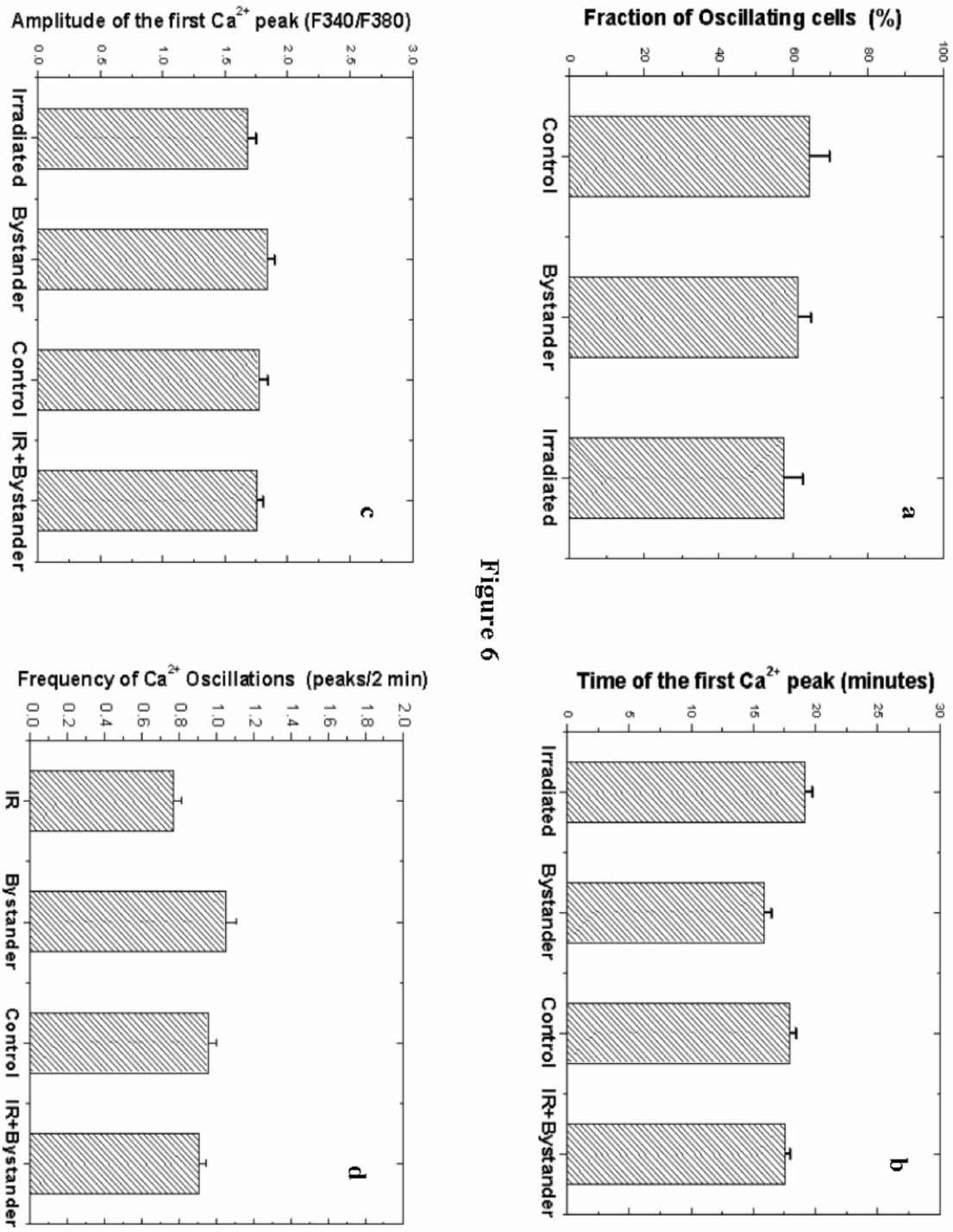
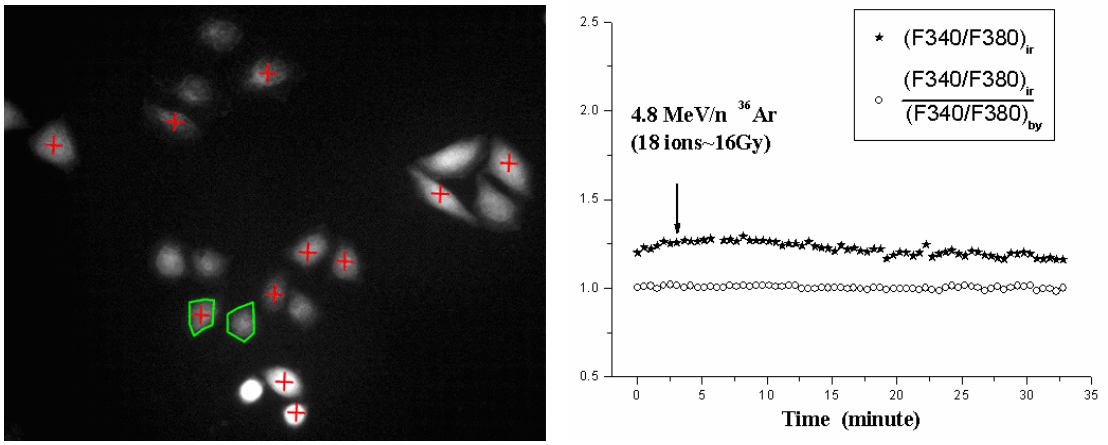


Figure 6

Fig. 6 Heavy ion irradiation has no significant effect on the stress-induced Calcium oscillations in AG1522-D cells in the standard cell dishes. (a) The fraction of the oscillating cells (type B cells) in the population of the carbon ion irradiated cells, non-irradiated bystander cells and control cells. (b-d) The onset (b) and the amplitude (c) of the first Ca^{2+} peak, and the frequency (d) of the Calcium oscillations in the spontaneously oscillating cells (64 irradiated, 61 bystander and 65 control cells). The data are plotted as mean ± standard

error of mean. No significant difference is found between cells in the control dishes and cells in the irradiated dishes (IR+Bystander). The difference between irradiated cells and bystander cells in (b-d) is due to the difference of their size and fluorescence brightness, normally large and bright cells were manually selected as targets to be irradiated.

In the same kind of experiments, the effect of ion irradiation was examined on HeLa cells in both standard and gas permeable cell dishes. Control experiments revealed that in contrast to fibroblast cells HeLa cells kept a stable resting $[Ca^{2+}]_{cyt}$ in both kinds of cell dishes. Fig. 7 shows a typical experiment in which HeLa cells were hit by 18 Argon ions (~16Gy). The data show that also in this case ion irradiation resulted in no perceivable change in the $[Ca^{2+}]_{cyt}$ level. The same absence of a Ca^{2+} response to ion irradiation was observed in 133 irradiated cells in a total of 7 independent experiments with Carbon or Argon ion irradiation of up to 50 Gy. Also in this case the non-irradiated bystander HeLa cells exhibited no perceivable change of $[Ca^{2+}]_{cyt}$ in all the experiments.



(a) (b)

Fig.7 Heavy ion irradiation did not induce perceivable change of $[Ca^{2+}]_{cyt}$ in HeLa cells. (a) In exponential growing colonies, 50% of the populations are chosen for irradiation, and the rest are chosen as bystander control cells. (b) HeLa cell shows no change of $[Ca^{2+}]_{cyt}$ before and after Argon ion irradiation. Closed star: the fluorescence ratio of an irradiated cell as a function of time; open circle: comparison of the fluorescence ratio between the outlined twin cells in (a), one was irradiated $(F340/F380)_{ir}$, the other not $(F340/F380)_{by}$.

Discussion

In previous experiments the $[Ca^{2+}]_{cyt}$ response of different cell lines has been examined under the influence of X-, γ- and β–radiations. In some cases it was demonstrated that different kinds of radiation evoked various types of Ca^{2+} responses namely oscillations, transient excursions and long lasting elevations (*3,7-9, 18-19*).

However, Hallahan et al. also reported that a treatment of human fibroblast cells and cancer cells even with a dose as high as 30 Gy β-radiation resulted in no perceivable effect on $[Ca^{2+}]_{cyt}$ (*20*). These reports provide no consistent picture on the behavior of $[Ca^{2+}]_{cyt}$ under ionizing radiation. The diversity of these results is not understood, but it is possibly related to the fact that the Ca^{2+} response to ionizing radiation depends on the cell types, the type of the radiation and the time of the monitoring.

The present work has the advantage that the addition of a ratio imaging setup into the single ion hit facility is a means of monitors $[Ca^{2+}]_{cyt}$ with high spatial and temporal resolution quasi in real time. In this way a single cell is not only irradiated in a defined area of the cell but the $[Ca^{2+}]_{cyt}$ of the same cell can be monitored continuously before and immediately after treatment and compared to that of adjacent non-irradiated control cells. The key observation of this approach is that irradiation of HeLa cells and AG1522-D cells with heavy ions results in no perceivable $[Ca^{2+}]_{cyt}$ change within seconds to half an hour after the treatment. The absence of a Ca^{2+} response is not due to the culture conditions, because control experiments using H_2O_2 as a stimulus confirm that cells in the same type of cell dish can respond to H_2O_2 by elevation of $[Ca^{2+}]_{cyt}$. Moreover, AG1522-D cells exhibited spontaneous $[Ca^{2+}]_{cyt}$ oscillations under the recording conditions; this clearly demonstrates that the cells are in principle capable of responding with elevation of $[Ca^{2+}]_{cyt}$ to stimuli. The spontaneous oscillations in $[Ca^{2+}]_{cyt}$ of AG1522-D cells were elucidated as responses to hypoxia stress in air tight cell dishes. But again such a hypoxia stress is not the reason for the absence of a Ca^{2+} response to ion irradiation, because the same type of cells did also not respond to ion irradiation in non-stressed cell dishes allowing a better gas exchange. In summary, our data show that irradiation with heavy ions has no effect on triggering Ca^{2+} signaling in the two cell lines tested.

DNA damage by DSB is formed directly upon clustered ionization in ion irradiation (*21*). The immuno-microscopy shows γ-H2AX foci, which label the phosphorylated histone H2A in response to DNA DSBs (*15*). This positive immuno staining of the same cells, which were monitored for their Ca^{2+} responses verifies the exact ion hits at the targeted positions (Fig. 3). In other words, our results show that DNA damage by double strand breaks did not induce any Calcium response in the time window of 30 minutes.

Todd et al suggest that the $[Ca^{2+}]_{cyt}$ response could depend on the state of the cell cycle; they observed a transient Calcium increase in S and G_2/M-Phase but not in G1-Phase HeLa cells exposed to β-radiation (*18*). In our experiment, about 130 exponentially growing HeLa cells were exposed to 0.1 to 50 Gy of heavy ion irradiation and monitored for their $[Ca^{2+}]_{cyt}$ change (see photo of HeLa colonies in the supplemental materials). With such a large number of analyzed cells one can expect that the cells under scrutiny comprise a statistical distribution of all cell cycle states. However, none of the cells examined shows any appreciable Ca^{2+} response. This suggests that the cell phase of HeLa cells doesn't play a role in the absence of Calcium response to heavy ion irradiation.

Calcium oscillations such as those induced by the stress are the result of a complex Ca^{2+} induced Ca^{2+} release (CICR) mechanism. This mechanism includes a number of coordinated steps of Calcium mobilization and buffering in a cell (*17*). The occurrence of these oscillations offers a possibility to investigate the influence of ion irradiation on the kinetic steps of CICR. As shown in Fig. 5b the irradiated AG1522-D cells show a similar behavior as those in the control experiment without any irradiation (Fig. 4). Also a detailed comparison of the oscillations in treated versus untreated cells revealed no irradiation associated impact on the oscillation. This suggests that ion irradiation has neither positive nor negative influence on any of the steps contributing to the complex mechanism of the CICR and the resulting oscillations. Hallahan et al also reported that Bradykinin-induced $[Ca^{2+}]_{cyt}$ increase was not inhibited by β-radiation (*20*). The fact that irradiation does also not effect the kinetic properties of the spontaneous oscillations further allows to conclude that irradiation has also no impact on the complex mechanism underlying Ca^{2+} induced Ca^{2+} release, i.e. the mechanism responsible for these oscillations.

The Carbon and Argon irradiation used in this study have a linear energy transfer (LET) of 300 and 2000 keV/µm. This is much higher than the LET of 0.2 to 12 keV/µm used in other related studies. It has been reported that cellular PKC responses could differ according to the LET (*22*). These authors found that high LET radiation had only a marginal effect on Protein Kinase C (PKC) mRNA expression while low LET radiation revealed a strong effect. Worth noting is that Calcium mobilization from internal stores and PKC activation share the same pathway via hydrolysis of phosphatidylinositol (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG) (*23-24*). In this context it might be possible that the absence of a Ca^{2+} response in HeLa and AG1522-D cells to ion irradiation is LET-dependent.

A large body of data has demonstrated that non-irradiated cells co-cultured with irradiated cells or cultured in medium from irradiated cells showed cellular damage response (*25-29*). This so-called ionizing radiation-induced bystander effect has been proposed to result from potential cell-to-cell signaling mechanisms via secreted factors or intercellular gap junctions, including calcium signaling (*30*). It was reported that irradiated cell conditioned medium (ICCM) induced Calcium fluxes in HPV-G and fibroblast cells (*31-33*). This implies the involvement of secreted factors, which are able in triggering calcium signaling. In addition, gap junctions constitute direct intercellular channels and allow the exchange of ions and small molecules, including Ca^{2+} and IP3 (*34*). Such cellular connections can also be indirectly confirmed from the present data because we have seen synchronized Calcium oscillations between fibroblast cells (Fig. 4c). However monitoring of $[Ca^{2+}]_{cyt}$ in non-irradiated bystander cells reveals in the present study no perceivable change in $[Ca^{2+}]_{cyt}$. This was independent on whether the bystander cells were in direct contact to irradiated cells or not. This implies that Calcium signaling neither plays a role in the early cellular response to ionizing radiation nor in the early stage of the bystander effect. Consequently, the mechanism of the ICCM induced elevation of Ca^{2+} (*31-33*) must be developed on a much longer time scale than that monitored here.

In conclusion, our experiments demonstrated that the ion irradiation and radiation-induced DNA DSBs, did not induce any response of $[Ca^{2+}]_{cyt}$ within seconds to half an hour after the treatment in human foreskin fibroblast cells and HeLa cells. This treatment also had no influence on the signaling network of Calcium oscillation induced by hypoxia stress.

Acknowledgements

This work was supported by the European Union under a Marie Curie Research Training Network Contract No. MRTN-CT-2003-503923. The authors thank B. Jakob and T. Pouthier for the help with immunostaining and confocal microscopy.

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